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Biochimica et Biophysica Acta 1783 (2008) 214–223

Biochimica et Biophysica Acta BBA

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Stringent time-dependent transregulation of calcium calmodulin kinase II (CaMKII) is implicated in anti-apoptotic control

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Received 30 May 2007; received in revised form 10 October 2007; accepted 11 October 2007
Available online 22 October 2007

Abstract

Induction of apoptosis by the PP1/PP2A inhibitor calyculin A was inhibited if the CaMKII inhibitor KN-93 was added no later than 10 min after addition of calyculin A. The physiological relevance and mechanism of CaMKII during apoptosis, however, remains largely unclear. Here we show in MDCK and gastric parietal cells that normal transregulation of CaMKII terminates the initial burst of autophosphorylation after only 10 min. The kinetics of CaMKII involved transregulation by PP1, PP2A, PP2B and PKCα. Transregulation of CaMKII resulted in two kinetic phases for phosphorylation of the autoactivation site at T286/287. During the initial phase, there was a clear peak of phosphorylation that lasted 10 min. This phase was subsequently followed by a half but constant level of T286/287 phosphorylation. Calyculin A perturbed this transregulation, resulting in a hyperphosphorylated CaMKII. This effect of CA on the kinetics of CaMKII was observed *in vivo* as well as *in vitro* using isolated tubulovesicles. Calyculin A-induced hyperphosphorylation of CaMKII appears to be at least one mechanism used by cells to trigger apoptosis. Therefore, stringent limitation of CaMKII autophosphorylation at T286/287 by transregulation and prevention of hyperphosphorylation seems to restrict apoptosis.

Keywords: Adaptation; Apoptosis; Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII); Proteinphosphatases; Protein kinase C (PKC); Signal transduction

1. Introduction

The Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) transduces elevated Ca^{2+}-signals to phosphorylate a number of target proteins [1,2]. Activation of CaMKII decodes the frequency of Ca^{2+}-spikes [3,4]. Upon Ca^{2+}/calmodulin-dependent activation, CaMKII undergoes cisphosphorylation at T286/287 of the autonomy site and T305/306 of the calmodulin binding site [3,4]. Phosphorylation at T286/287 corresponds with activated CaMKII [1–3]. Several phosphoprotein phosphatases such as PP1, PP2A and PP2C are known to dephosphorylate at least phospho-T286/287, which reverses the autonomous state and limits the activity of CaMKII [5–7].

Overexpression of CaMKII was shown to increase apoptosis [8]. Inhibition of CaMKII-counteracting phosphatases PP1 and PP2A by microcystin also demonstrated that CaMKII is involved in apoptosis [8]. CaMKII has been implicated in distinct phases of apoptosis [9]. An early effect ($t_{0.5}$=9 min) of microcystin was shrinkage of cytoplasmic and nuclear volume, which could be reversed by the addition of a CaMKII inhibitor. Microcystin-dependent dysregulation of CaMKII for 14 min was necessary to induce the loss of plating ability and the start of budding in 50% of cells [9]. At an early time-point, microcystin-induced apoptosis was reversed by specific CaMKII inhibitors, whereas after 15 to 17 min microcystin induced cell death could not be abolished by inhibition of CaMKII [9]. For both microcystin (0.5 to 2 μM)-induced effects, membrane budding and the inability to attach to a substratum, CaMKII activity was necessary for 11.5 to 16.5 min [9]. After 31 to 40 min of microcystin application, chromatin hypercondensation could not be abolished by inhibition of CaMKII [9]. These data suggest that CaMKII should be regulated in such a way as to prevent its induction of apoptosis but allow for CaMKII-dependent physiological processes. Epithelial CaMKII,

Abbreviations: CA, calyculin A; SA, secretory apical membrane; TV, tubulovesicles

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doi:10.1016/j.bbamcr.2007.10.005

for instance, is necessary to regulate the calcium-induced function associated with exocrine acid secretion [10,11].

Here, we demonstrate that time-dependent transregulation of CaMKII activity appears to protect cells from apoptosis. Of general interest, CaMKII is thought to be a target for drug therapy to prevent CaMKII-dependent apoptosis [12]. Therefore, knowledge of the cellular kinetics of CaMKII may aid the design of an apoptosis-limiting therapy without affecting other CaMKII-dependent physiological processes.

2. Materials and methods

2.1. Primary cell culture and preparation of parietal cell membranes

Parietal cells of gastric mucosa were isolated from the stomachs of male "Wistar" rats by adapted protocols according to [10,13,14]. Preparations of resting state apical membrane and tubulovesicles (TV) as well as carbachol (10^{-4} M)-stimulated apical membrane (SA) from the homogenate of rat gastric mucosal cells were performed as described [10]. Gastric mucosal cells containing parietal cells were isolated from PKCα(+/+) or PKCα(−/−) mice, and cultured for a short time in DMEM F-12 medium. PKCα mutant mice were generated as previously reported [15]. The enriched membrane fraction of carbachol (10^{-4} M)-stimulated cells from mice was regarded as vesicles similar to SA. Limited amounts of gastric mucosa from PKCα(−/−) mice prevented preparation of sufficient amounts of TV in parallel.

2.2. Transient transfection and confocal microscopy

MDCK cells have been used for transfection as gastric parietal cells demonstrate a limited rate of transfection. MDCK-II cells, very generously supplied by Dr. Beate Sodeik (Institute of Virology, Medizinische Hochschule Hannover, Germany), were transiently transfected utilizing various forms of CaMKII, each of which was C-terminally fused to mGFP. This is a mutated form of eGFP at A207K to abolish aggregation of GFP [16]. All mGFP-CaMKII constructs were very kindly provided as plasmids by Prof. Paul De Koninck (Université Laval, Québec, Canada). In the text, mGFP is abbreviated as GFP unless mentioned otherwise. The following various mutant forms of CaMKII were generated as described previously: 286Ala [17], 286Asp [18], 1–326 [3] or 315–478 [19]. GFP-CaMKII exhibited phosphotransferase activity [20]. Mutant forms of the kinase were subcloned into the GFP-CaMKII wild type backbone using a *PflM*I restriction insert [20]. Transfections were performed with lipofectamin 2000 in Optimem medium (both Invitrogen, Carlsbad, CA) with subsequent culture in DMEM containing high glucose (4.5 mg ml^{-1}). Green fluorescence of live cells was monitored with a Leica TCS SP2 AOBS confocal LASER scan microscope (Leica Microsystems, Heidelberg, Germany) at 488 nm excitation to determine the rate of transfection, which was ≥80%. GFP-CaMKII expression was also monitored with an anti-GFP antibody (sc-8334; Santa Cruz Biotechnology, Santa Cruz, USA).

2.3. Assays for cytotoxicity

Cytotoxicity of calyculin A (CA) on gastric parietal cells was determined as apoptosis or necrosis. KN-93 was left on cells for 2 h, which were subsequently washed with medium. Apoptosis was measured by assessing DNA degradation utilizing the "Cell Death Detection ELISA^{plus} kit" (Roche, Mannheim, Germany) 24 h after induction. This ELISA is based on the quantitative detection of mono- and oligonucleosomes in the supernatant of lysed cells using specific mouse monoclonal antibodies. In parallel, release of lactate dehydrogenase (LDH) was determined by utilizing the "Cytotoxicity kit" from Roche.

2.4. Determination of CaMKII phosphorylation

SDS-polyacrylamide gel electrophoresis and immunoblot procedures were performed as previously described [10]. To determine the effect of phosphatases on CaMKII and since CaMKII uses itself as a substrate (cisphosphorylation), the CaMKII regulatory state was measured as autonomy site-phosphorylated CaMKII [3] instead of external substrate phosphorylation. As phosphatases may not only dephosphorylate phosphorylated CaMKII but also CaMKII-phosphorylated substrates, an external substrate was not used. A ternary complex of CaMKII, phosphatase and a substrate of CaMKII may exhibit various states of phosphorylation and dephosphorylation. This would be much more difficult to analyze than the herein analyzed phosphorylation sites of CaMKII. Phosphorylation of the CaMKII autonomy site was detected with a rabbit polyclonal anti-phospho-T286/287 CaMKII(rat) antibody (Promega, Mannheim, Germany). There is a strong correlation between the state of autoregulatory phosphorylation of CaMKII and its phosphotransferase [1–3,10]. Phosphorylation at T305/306 of CaMKII was monitored with a rabbit polyclonal anti-phospho-T305/306 CaMKII(rat) antibody (Chemicon/Millipore, Schwalbach, Germany). To imitate the cellular Ca^{2+} influx as local, rapid Ca^{2+} peaks, Ca^{2+}(0.1 mM) input for full activation of exocrine CaMKII [21] was constant during *in vitro* analysis of CaMKII signalosomes. As a standard reference, CaMKII was purified from hog gastric mucosa to apparent homogeneity as previously reported [22]. For pharmacologic or toxic agents, the following final concentrations were used unless stated otherwise: Carbachol (10^{-4} M; Sigma, Munich, Germany), calyculin A (10^{-8} M; Calbiochem, Nottingham, UK), FK 506 (10^{-7} M; Alexis, Lausen, Switzerland), Gö 6976 (10^{-5} M; Calbiochem), KN-62 (5×10^{-5} M; Calbiochem), KN-93 (5×10^{-5} M; Calbiochem), okadaic acid (10^{-8} M; Calbiochem), ranitidine (10^{-4} M; Sigma), Ro 31-8220 (10^{-7} M; Calbiochem) and TPA (10^{-7} M; Calbiochem). Autoluminographs were densitometrically analyzed. Data values for intensities were normalized to control.

2.5. Statistical analysis

Data values are presented as means±standard error of means (S.E.M.). Data of at least three groups were analyzed for variance (ANOVA) followed by a *post hoc* Dunnett test. $P<0.05$ was considered to be significant.

3. Results

3.1. Apoptosis is blocked by KN-93 for up to 10 min after induction by CA

To determine the CaMKII-dependent and -independent phases of CA-induced apoptosis, MDCK cells were incubated with CA (10 nM) for various periods of time ranging between 0 and 30 min. After incubation, cells were washed and incubated either with or without the specific CaMKII-inhibitor KN-93 (50 μM) (Fig. 1a). To monitor the effect of KN-93 (50 μM) alone, it was added at the indicated timepoints between 0 and 30 min. There was a time range between 10 and 11 min in which CA-induced apoptosis shifted from total sensitivity to insensitivity of KN-93. (Fig. 1a). Carbachol (0.1 mM)-stimulated gastric parietal cells were used to compare the effects of CaMKII-inhibitors KN-62 and KN-93 after 10 min or 30 min induction of apoptosis by CA (10 nM). The CaMKII inhibitors demonstrated a similar ability to block the CA-induced apoptosis after 10 min, and a failure to block after 30 min (Fig. 1b). Experiments were performed as for MDCK cells (Fig. 1a). 5.53±1.7% of untreated parietal cells exhibited spontaneous apoptosis.

Each GFP-CaMKII vector construct was tested for its effect on apoptosis (Fig. 1c) and LDH release (Fig. 1d) in MDCK cells. Transfection of WT GFP-CaMKII or the association domain truncated GFP-CaMKII 1–326 increased the apoptotic rate 1.4±0.2 and 1.7±0.08-fold, respectively (Fig. 1c). The association domain alone (GFP-CaMKII 315–478) resulted in a 2.9±0.03-fold higher rate of apoptosis. Mutated forms of the T286 CaMKII autonomy site (GFP-CaMKII T286A or T286D),

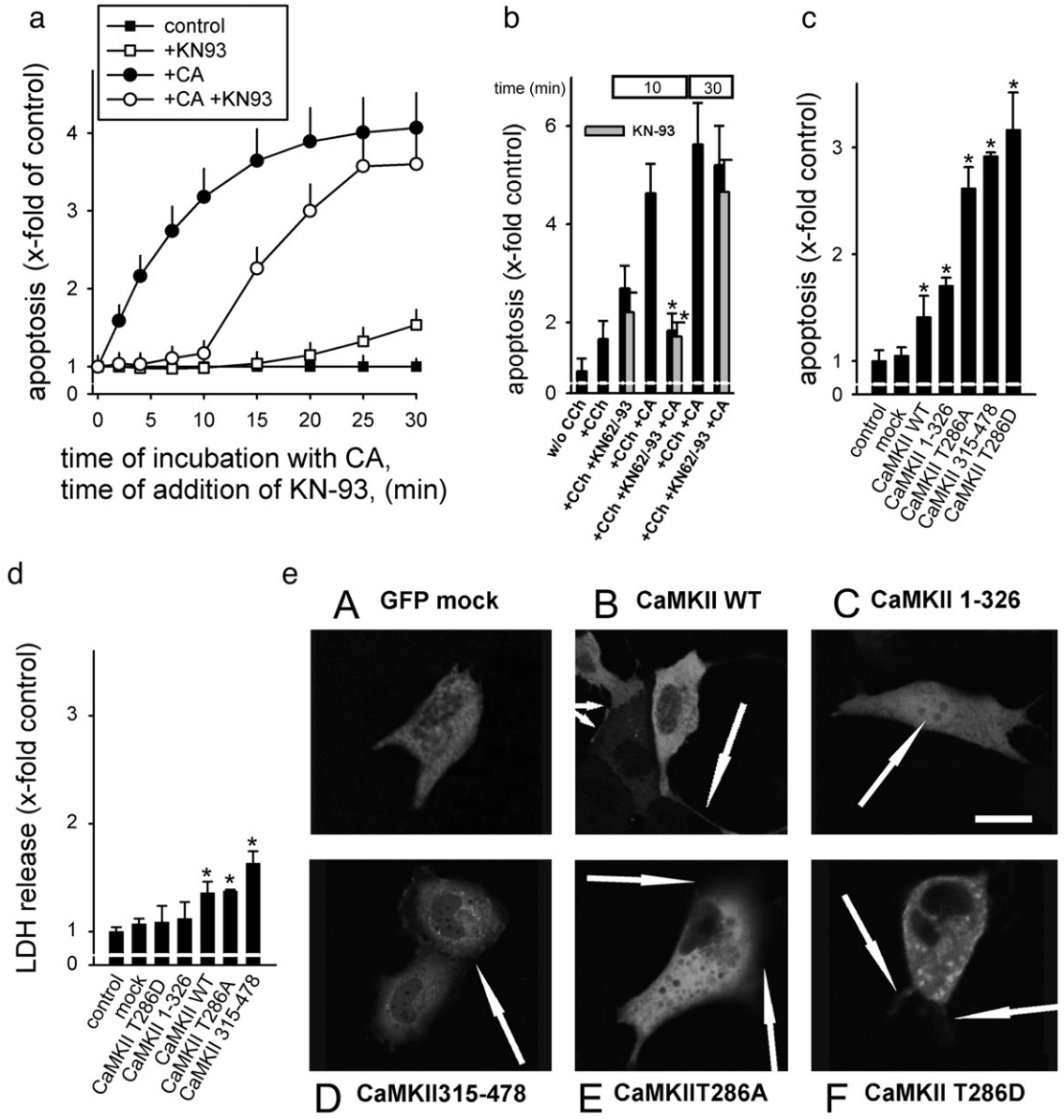
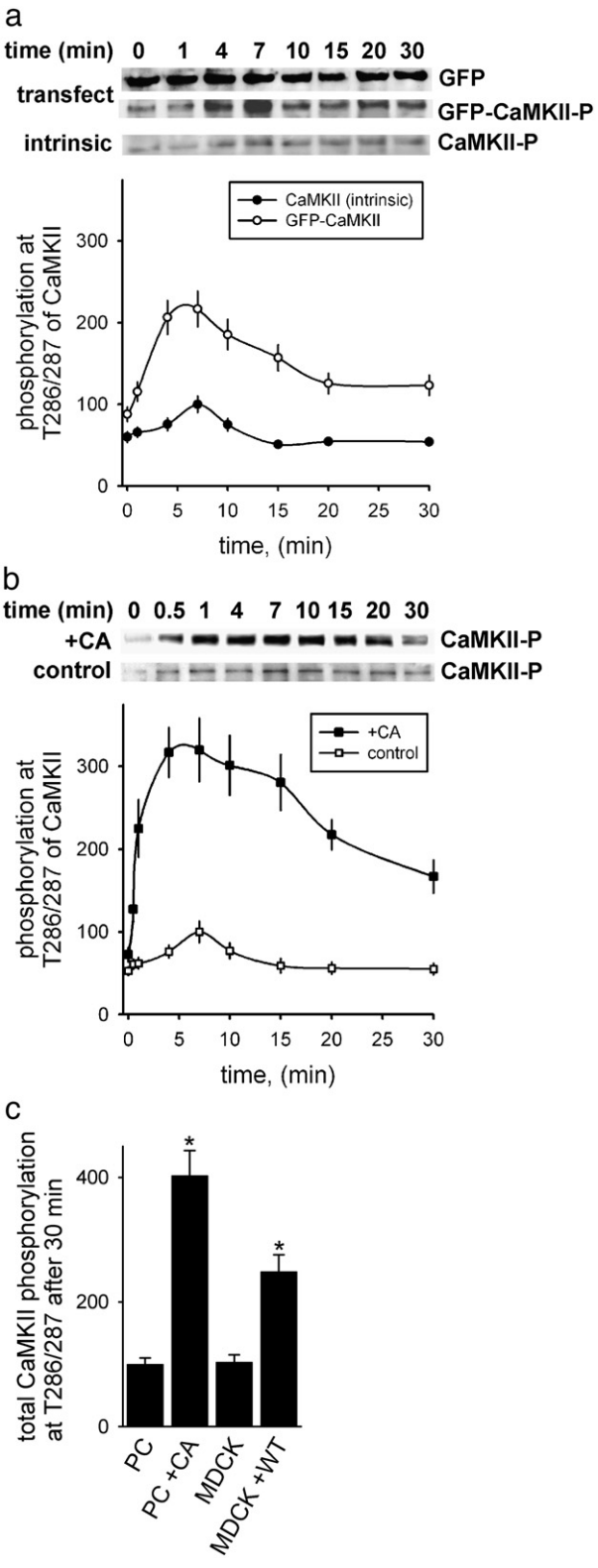


Fig. 1. Physiologically regulated CaMKII prevents apoptosis only 10 min. (a) MDCK cells were treated with CA (10 nM) for various time ranges between 0 and 30 min (black points). To demonstrate the effect on CaMKII, KN-93 (50 μM) was added directly in exchange for CA at the given timepoints (0 to 30 min) (white-filled circles). As controls, cells were left either untreated (black squares) or incubated with KN-93 (50 μM) alone, which was added at the given timepoints (white-filled squares). KN-93 was left on cells for 2 h. To stimulate CaMKII, ionomycin (1 ng ml^{-1}) was present in each assay. Apoptosis was determined after 24 h of CA treatment by release of oligo- and mononucleosomes using an ELISA. (b) The same experimental conditions as used for MDCK cells in (a) were examined for gastric parietal cells except that parietal cells were stimulated with carbachol (CCh, 0.1 mM) instead of ionomycin. Parietal cells were incubated with CA (10 nM) for 10 or 30 min. CA ("+CCh+CA") was then exchanged against cell medium without CA, or with KN-62 (50 μM) or KN-93 (50 μM) ("+CCh+KN62/−93+CA"), respectively. As controls, no carbachol-induction ("w/o CCh"), no toxins ("+CCh"), or only KN-62 or KN-93 ("+CCh+KN62/−93") was added after 10 or 30 min of CCh-stimulation. *, significant differences to CCh+ CA alone, $P<0.05$ or less. (c) MDCK cells were transiently transfected with various mutated forms of GFP-CaMKII. 24 h after transfection, apoptosis was determined by mono- and oligonucleosomes. Different forms of CaMKII with a mutated autonomy site (GFP-CaMKII T286A, T286D) as well as the association domain alone (GFP-CaMKII 315–478) showed significantly increased rates of apoptosis ranging between 2.6 and 3.2. (d) MDCK cells were transiently transfected with various mutated forms of GFP-CaMKII. 24h after transfection, LDH release was determined. LDH release for the same set of transfections as used for the determination of apoptosis was only minimally increased. *, significant differences in the corresponding control, $P<0.05$ or less. (e) Mutant forms of CaMKII modified cell shape and visible cell–cell contact. MDCK cells were transiently transfected with mutant forms of CaMKII, each of which was fused to GFP, and monitored in living cells which were not part of a epithelium-like monolayer of cells. (A) Expression of GFP alone without fused CaMKII. (B) Transfected wild type GFP-CaMKII shows expression over the whole perikaryon as well as the branches (long arrow). Cell–cell contact zones became visible as a result of different levels of GFP-CaMKII expression (short arrows). (C) GFP-CaMKII 1–326 was localized to the nucleus (arrow) to a much greater extent than the rest of the cell. (D) Expression of the GFP-tagged C-terminal association domain alone (CaMKII 315–478) led to rounded cell shape and visibly loose cell–cell contact (arrow). (E) Cells with overexpressed GFP-CaMKII T286A show numerous vacuoles. The body of the cell is rounded up at the nucleus-bearing side of the cell (arrows). (F) Expression of GFP-CaMKII T286D became punctuate throughout the cytosol and poorly expressed in shortening branches (arrows). Scale bar, 20 μm.

which cannot be phosphorylated, showed a 2.6±0.2- or 3.2±0.35-time increased rate of apoptosis, respectively. GFP vector ("mock") alone did not cause apoptosis (Fig. 1c). Each LDH assay of various transfected GFP-CaMKII mutant forms showed only minor increases of LDH release (necrosis) (Fig. 1d).



3.2. Mutant forms of unregulated CaMKII modify cell shape

MDCK cells were transiently transfected with mutant forms of CaMKII fused to GFP [20] for monitoring by live cell imaging. GFP expression alone ("GFP mock") showed a diffuse distribution of GFP throughout the cell (Fig. 1e-A). Wild type CaMKII also showed diffuse expression throughout the cell body as well as in branches (Fig. 1e-B, long arrow). Cell–cell contact zones became visible by expression of WT GFP-CaMKII (short arrows, Fig. 1e-B). Expression of GFP-CaMKII 1–326, which does not contain the association domain, tended to induce a stretched shape in cell, and was abundant throughout the cell, although was predominant in the nucleus (arrow) (Fig. 1e-C). Expression of the GFP-tagged association domain alone (CaMKII 315–478) led to a completely rounded cell shape and a visible loss in cell–cell contact (arrow) (Fig. 1e-D). GFP-CaMKII T286A showed a diffuse distribution in cells with numerous vacuoles. The cell body was rounded up at the nucleus-containing site (arrows) (Fig. 1e-E). Expression of GFP-CaMKII T286D became punctuate throughout the cytosol and was poorly expressed in short branches (Fig. 1e-F, arrows). Cells were remarkably round shaped (Fig. 1e-F).

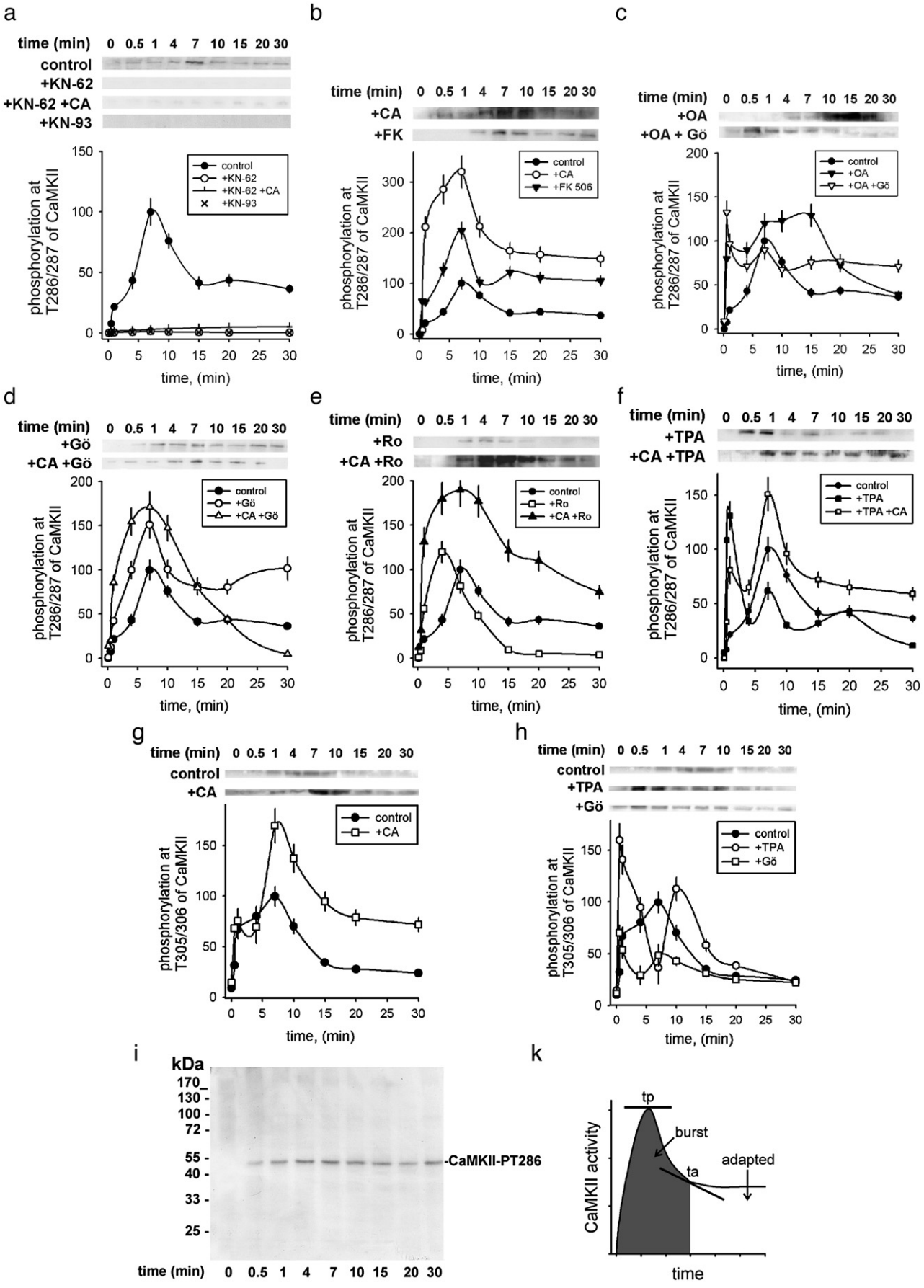
3.3. Kinetics of CaMKII phosphorylation

Overexpression of GFP-CaMKII in MDCK cells led to higher phosphorylation of CaMKII at T286/287 than intrinsic CaMKII induced by ionomycin (1 ng ml^{-1}) (Fig. 2a). There was, however, a much higher expression of transfected GFP-CaMKII than of intrinsic CaMKII. The latter could not be detected with an anti-non-phospho CaMKII antibody (data not shown). GFP-CaMKII was clearly expressed, as shown by fluorescence in live cells (Fig. 1d) and by immunoblot staining with an anti-GFP antibody (Fig. 2a). Each intrinsic or overexpressed CaMKII showed kinetics of autonomy site phosphorylation with a maximum after 7 min. Treatment of carbachol (0.1 mM)-stimulated gastric parietal cells with CA (10 nM) resulted in 4-fold higher total phosphorylation at T286/287 than the control (Fig. 2b, c). A preliminary comparison of total phosphorylation at T286/287 of CaMKII overexpressing cells ("MDCK+WT") to CA-treated parietal cells ("PC+CA") showed 1.6±0.03-fold higher phosphorylation in the latter (Fig. 2c).

3.4. Effect of transregulators on the phosphorylation of TV-signalosome-integrated CaMKII

As a reference to non-signalosome-integrated CaMKII, purified CaMKII showed a continuous increase of autonomy

Fig. 2. *In vivo* kinetics of phosphorylation of CaMKII autonomy site T286/287. (a) MDCK cells were analyzed for phosphorylation of the autonomy site on either intrinsic CaMKII or transfected GFP-CaMKII WT after treatment with ionomycin (1 ng ml^{-1}). (b) Gastric parietal cells (PC) were stimulated with carbachol (0.1 mM) either in the absence or presence of CA. Phosphorylation of CaMKII at the autonomy site was analyzed at each timepoint indicated. (c) Total phosphorylations at T286/287 of CaMKII between 0 and 30 min of experiments presented in (a) and (b). Data show the mean±S.E.M.*, significant differences to the corresponding control, $P<0.05$ or less. $n=3–6$ separate experiments.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+KN-93
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+FK
phosphorylation at T286/287 of CaMKII
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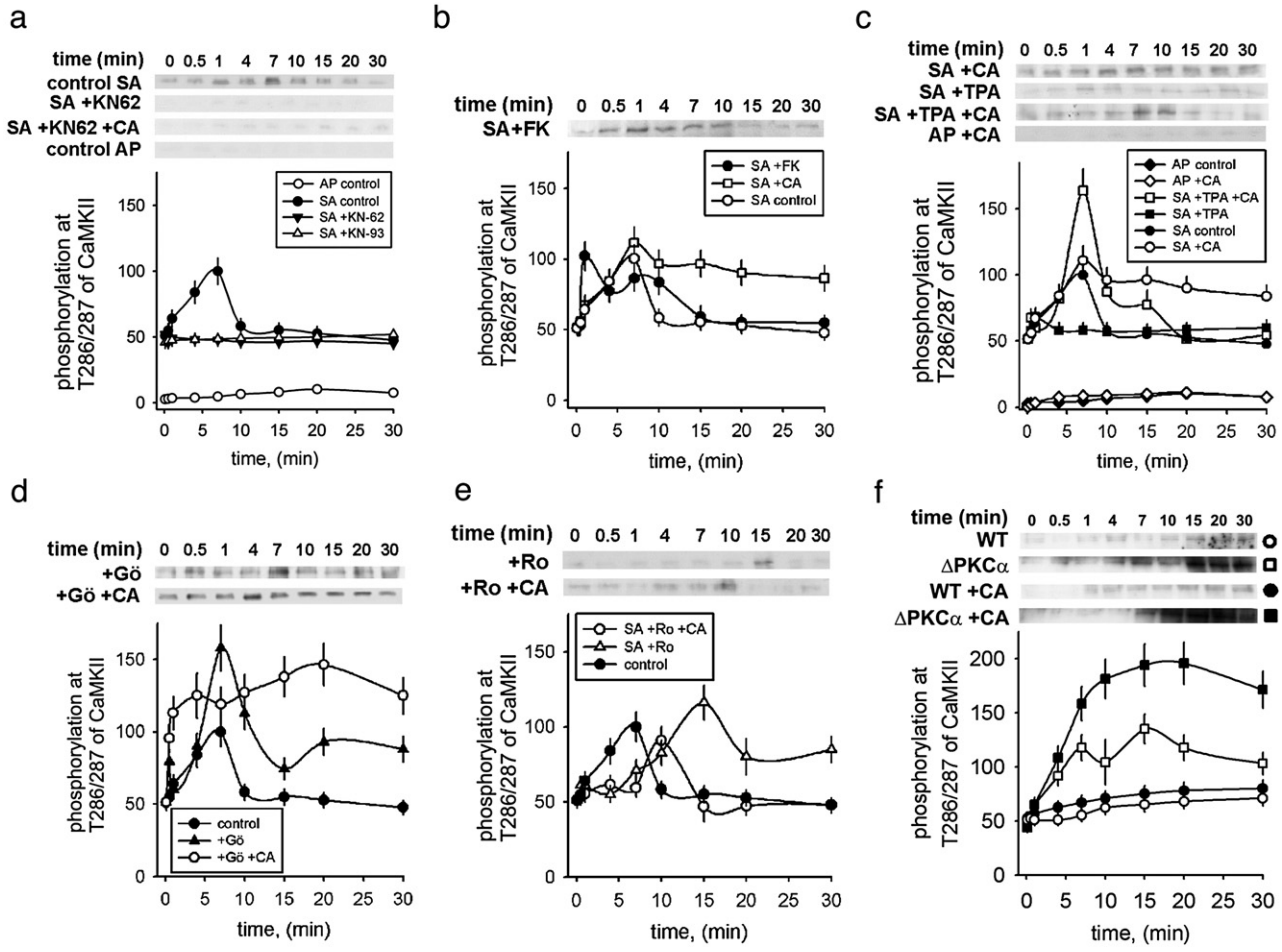

Fig. 4. Effect of various protein kinase modulators and phosphatase inhibitors on the kinetics of specific phosphorylation of CaMKII T286/287 of SA. Specific CaMKII phosphorylation utilizing an anti-phospho T286/T287 was normalized to 100% for maximum phosphorylation in each control assay. Kinetics of phosphorylation were performed with $n=3$ to 6 different experiments. (a–f) control without toxins, (a) KN-62, KN-93, (b–f) CA, (b) FK 506, (c) TPA, (d) Gö 6976, (e) Ro 31-8220. (f) Increased phosphorylation of CaMKII T286/287 present in SA-like plasma membrane of PKCα-deficient mice. Gastric mucosal cells containing exocrine parietal cells from either wild type (WT) mice or PKCα(−/−) knockout mice (ΔPKCα) were short-term cultured and stimulated with carbachol (0.1 mM) in the presence of ranitidine (0.1 mM) for 10 min, and optionally CA. For concentrations of toxic agents, see Materials and methods. The standard error of the means is indicated by error bars.

site phosphorylation without adaptation (data not shown, see [21]). For CaMKII in its TV signalosome, control without any modulator showed a transient peak of phosphorylation followed by adaptation to half peak but constant autonomy site phosphorylation at T286/287 between 4 and 15 min (Fig. 3a). KN-93 as well as KN-62 strongly inhibited CaMKII phosphorylation (Fig. 3a). This effect was not compensated or reversed by CA. CA alone resulted in a strong increase in CaMKII burst phosphorylation designated "hyperphosphorylation" with adaptation on a more than 3-fold higher level than the control (Fig. 3b). Maximal peak phosphorylation of T305/306 was only half compared to T286/287 when treated with CA (Fig. 3g). Okadaic acid, which is 100-fold more selective for PP2A than PP1, prolonged the duration of the initial, hyperactive phase from 11 min to 25 min compared to control (Fig. 3c). The PP2B-inhibitor FK 506 had similar but lower effects than CA on CaMKII phosphorylation (Fig. 3b). The cPKC inhibitor Gö 6976 shifted the maximum peak of autonomy site phosphorylation as well as the adapted phosphorylation to a higher level compared to control (Fig. 3d). Remarkably, and in contrast to phosphorylation of

Fig. 3. Effect of various protein kinase modulators and phosphatase inhibitors on the kinetics of specific CaMKII-phosphorylations in its TV signalosome. Specific CaMKII phosphorylations utilizing appropriate antibodies (anti-phospho T286/T287 (a–f) or anti-phospho T305/306 (g, h)) were normalized to 100% for maximum phosphorylation in each control assay. Insets display typical immunoblots of autonomy site-specific phosphorylated CaMKII. Kinetics were performed with $n=3$ to 6 different experiments. (a–h) Control without toxic agents, (a) KN-62, KN-93, (a+b, d–g) calyculin A (CA), (b) FK 506 (FK), (c, d, h) Gö 6976 (Gö), (e) Ro 31-8220, (f, h) TPA, (c) okadaic acid (OA). For the concentrations of toxins used, see the first paragraph of Results. (i) Full-length immunoblot to demonstrate the specificity of the anti-CaMKII phospho-T286/287 signal used to assess the kinetics after induction with Ca^{2+}. (k) Model of kinetics to illustrate the terms "burst" and "adapted" phase of phosphorylation of CaMKII. "t_p" is the timepoint of peak phosphorylation after Ca^{2+}-induction. "t_a" is the turning point that separates the burst from the adapted phase.

T286/287, the phosphorylation of T305/306 was clearly attenuated in the presence of Gö 6976 (Fig. 3h). Parallel blocking of PP1, PP2A and PKCα by CA and Gö 6976 resulted in an initially hyperphosphorylated CaMKII followed by a dampened phase to total loss of T286/287 phosphorylation. In the presence of the cPKC/nPKC inhibitor Ro 31-8220, which should be specific to PKCα as TV do not carry PKCε [11], an attenuated kinetics was observed in the presence as well as the absence of CA (Fig. 3e). The active phorbolester TPA used to activate PKC induced an attenuated oscillation-like phosphorylation of CaMKII at T286/287 and T305/306, each of which exhibited a main peak after 1 min of induction. A second but lower peak after 7 and 10 min of induction was observed for phosphorylation at T286/287 and T305/306, respectively (Fig. 3f, h). The specificity of the anti-CaMKII phospho-T286/287 is shown in Fig. 3i. A schematic diagram illustrates the two main phases, "burst" and "adapted", of the kinetics of TV-CaMKII phosphorylation. "t_p" is the time point of maximal phosphorylation after Ca^{2+}-induction (Fig. 3k).

3.5. Effect of transregulators on the phosphorylation of SA-signalosome-integrated CaMKII

The apical membrane of unstimulated, resting state cells exhibited a poor level of CaMKII T286/287 phosphorylation (Fig. 4a). CaMKII of stimulus-associated membrane (SA) displayed a basic level of phosphorylation at T286/287. Free Ca^{2+} induced a burst of T286/287 activation with a peak after 7 min. Adapted phosphorylation at T286/287 (15 to 30 min) was similar to that observed at timepoint 0 (Fig. 4a). The presence of CaMKII inhibitors KN-93 or KN-62 blocked the Ca^{2+}-induced burst of phosphorylation at T286/287. Moreover, T286/287 phosphorylation of TV-CaMKII (15 to 30 min) was in the same range as phosphorylation of SA-CaMKII at 0 min (Figs. 3a, 4a).

For SA, inhibition of PP1/PP2A by CA did not increase the initial burst of specific CaMKII T286/287 phosphorylation but elevated the adapted level compared to control (Fig. 4b). Combining Gö 6976 with CA strongly increased SA-CaMKII T286/287 phosphorylation without any clear adaptation (Fig. 4d). FK 506 did not alter the burst or adapted level of CaMKII phosphorylation compared to control (Fig. 4b). TPA inhibited bursting of SA-CaMKII phosphorylation *in vitro* (Fig. 4c). Gö 6976 increased the burst and adapted level of CaMKII T286/287 phosphorylation compared to control (Fig. 4d). Ro 31-8220 shifted the peak of maximum T287/287 phosphorylation from 7 to 15 min. Addition of both CA and Ro 31-8220 induced a shift in peak phosphorylation from 7 to 10 min (Fig. 4e).

For wild type mice, autonomy-site phosphorylation at T286/287 of CaMKII associated with the SA-like plasma membrane of carbachol-stimulated cells showed only a moderate increase in the presence of CA, suggesting a similarity to SA of rats (Fig. 4a, f). A clear increase in specific phosphorylation at T286/287 of SA-like associated CaMKII in carbachol-stimulated gastric mucosal cells was observed for PKCα(−/−) mice. This effect was enhanced in the presence of CA (Fig. 4f). Specific CaMKII activation at T286/287 at time point t_p=15 min was 1.8-fold higher in PKCα(−/−) mice in the absence, and t_p=17.5 min 2.5-fold higher in the presence of CA compared to wild type mice (Fig. 4f).

Comparing total phosphorylations of CaMKII T286/287 in the presence of various toxins, the SA signalosome was more robust than the TV signalosome, especially during the pre-peak phase (t_0 to t_p) (Fig. 5a, b). For CaMKII of SA-like membranes of PKCα-deficient mice, the pre-peak total phosphorylation was higher than that observed for rat SA. This pre-peak phase of PKCα-deficient mice was not as robust as rat SA in response to CA (Fig. 5a, b).

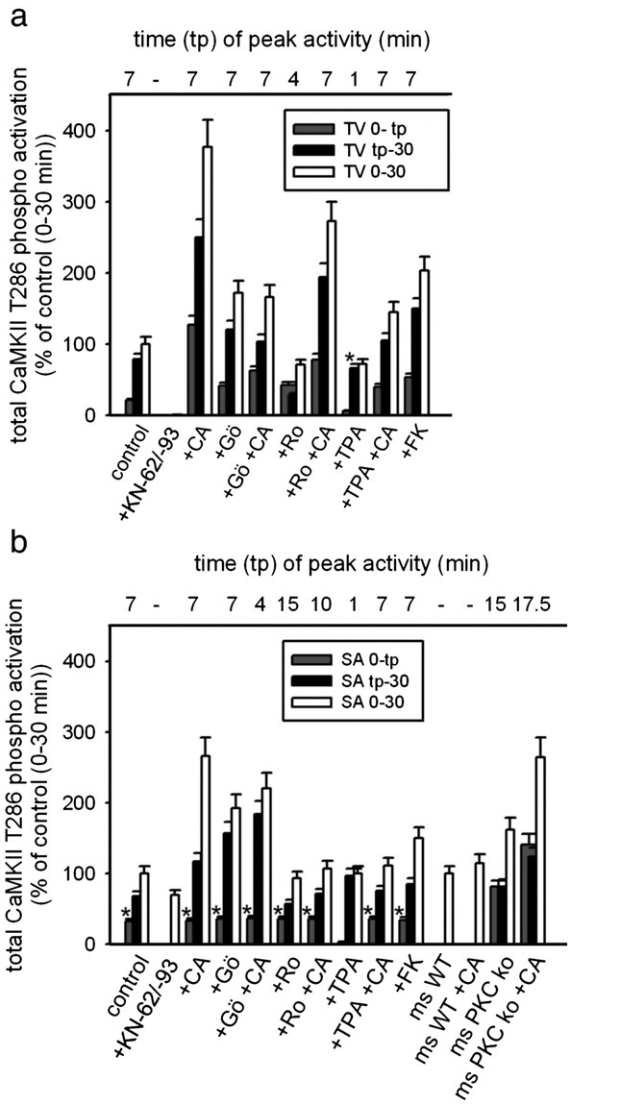

Fig. 5. Comparison of the robustness of autonomy site-specific total phosphorylation of the CaMKII signalosome in response to kinase or phosphatase modulators. Total autonomy site phosphorylation at T286/287 was compared between burst phase (time range between induction with free calcium at t_0=0, and time t_p of maximal peaking, grey bars, "0-peak"), and adaptation phase (time t_p after peaking to t_i=30 min after calcium induction, black bars, "peak - 30"). Individual timepoints of peaking after induction (t_p) are indicated at the upper *x*-axis. Total activations between t_0 and t_i are indicated by white coloured bars ("0–30"). (a) rat TV, (b) rat SA and mouse (ms) SA-like. All data are significantly different from the corresponding control ($P<0.05$) excluding (*), where no significance relative to control was observed. For abbreviations, see legend of Fig. 3.

4. Discussion

Epithelial CaMKII is a pivotal regulatory enzyme in exocrine calcium-dependent signaling [10] and, presumably, in apoptosis [8,9]. However, it is still unclear by which regulatory processes and in response to what conditions CaMKII switches from promoting exocytosis to inducing of apoptosis. As previously reported, inhibitory toxins of PP1/PP2A need an active CaMKII to induce apoptosis [8,9]. As PP1 and PP2A counteract the regulation of CaMKII [6,7,23], inhibition of PP1/PP2A should affect CaMKII regulation. We recently demonstrated that CaMKII is integrated in distinct signalosomes comprising at least PP1, PP2A, PP2B and PKCα [11]. This suggests a more complex regulation beyond CaMKII autoregulation [1–4]. It was also recently shown that CaMKII was implicated in different time-dependent phases of apoptosis [9]. To date, the kinetics of the regulatory phosphorylations of epithelial CaMKII present in signalosomes and its effect on apoptosis have not been addressed. Therefore, the time-dependent phosphorylation of CaMKII present in signalosomes and its effect on apoptosis were studied.

It was initially observed that treatment of cells with the phospho-serinyl/-threoninyl phosphatase inhibitors microcystin-LR, nodularin or okadaic acid increased apoptosis [8]. CaMKII was also shown to be involved in specific phases of microcystin-induced apoptosis in hepatocytes [9]. In the same study, it was proposed that inhibition of phosphoprotein phosphatases led to a more active CaMKII compared to cells not treated with phosphatase inhibitors. Both the inhibition of phosphoprotein phosphatases and the increased activity of CaMKII may lead to an accumulation of reactive oxygen species (ROS), resulting in increased apoptosis [9].

We confirmed that overexpression of a phosphomimetically activated CaMKII (CaMKII T286D) increased apoptosis in MDCK cells. This effect was also shown by Fladmark et al. for COS cells [8]. We did not, however, find a strong increase in apoptosis by overexpression of wild type CaMKII as demonstrated for COS cells [8]. Despite a high transfection rate, overexpressed WT CaMKII did not reach the level of T286/287 phosphorylation observed for intrinsic CaMKII in non-transfected MDCK cells treated with CA. The lower level of WT CaMKII activity in transfected cells compared to CA-treated cells does not seem to be sufficient to trigger apoptosis. In contrast, we found an apoptosis-inducing effect in response to either autonomy site non-phosphorylatable CaMKII T286A or constitutively active T286D as well as with the CaMKII association domain alone. We speculate that even overexpression of phosphorylation adjustable forms of CaMKII (WT, 1–326) but not regulation-deficient forms of CaMKII (T286D, T286A) can be integrated in CaMKII-carrying signalosome resulting in sufficient transregulation and subsequent prevention of hyperphosphorylation and apoptosis. The rounded shapes of MDCK cells transfected with regulation-deficient forms of CaMKII are indicative of an apoptotic phenotype. As these mutant forms of CaMKII increased LDH leakage only minorly, CaMKII alone does not seem to be involved in necrosis. Apoptosis was blocked by inhibition of CaMKII with KN-93 for up to 10–11 min after induction with CA. This suggests that CaMKII has to be active for at least 10 to 11 min to commit CA-induced apoptosis.

The question arises as to how the intracellular activity of CaMKII is organized to forward physiological tasks but prevent apoptosis. For gastric parietal cells, it was shown that CaMKII activity is functionally partitioned in time and space between cytoplasmic TV and SA [11]. Translocating TV are suggested to be the main source of active CaMKII at SA [10,11,24]. The concept of functional partitioning of CaMKII may be important besides to organize of the exocrine response, to control apoptosis. Thus, in the present study we here characterized cytoplasmic TV and SA for the kinetics of regulatory phosphorylation of CaMKII. We showed that Ca^{2+}-dependent kinetics of CaMKII in its signalosome exhibited an initial burst of phosphorylation at T286/287 as well as T305/306 followed by a medium level of "adapted" activity. This kind of kinetics was described 25 years ago as an adaptation process. [25]. Autophosphorylation activity at T286/287 correlates with phosphotransferase activity of CaMKII at TV or SA towards the CaMKII-specific, synthetic peptide substrate autocamtide-II [10,24,26].

PP1 and PP2A were important transregulators of CaMKII in the TV signalosome. In the late phase (20 to 30 min) of the examined kinetics of the TV-signalosome, and by comparing the effects of the PP1/PP2A inhibitor CA and PP2A-inhibitor okadaic acid, PP2A lost its effect on CaMKII phosphorylation. This may be explained by the fact that CaMKII and PP2A mutually downregulate each other. We propose that a CaMKII–PP1–PP2A system is predominantly responsible for the adaptation of CaMKII activating phosphorylation [27–29]. PP2B has been detected in the cytoplasm and nucleus, but not the plasma membrane, of gastric parietal cells [30]. PP2B is not known to dephosphorylate CaMKII directly [6], yet it supports the attenuation of cytoplasmic CaMKII activity presumably by upregulating the activity of PP1 indirectly by dephosphorylating its inhibitor phospho-I-1 [27].

A direct attenuator of CaMKII activity, presumably by direct phosphorylation, is PKCα. This was shown on the biochemical level with purified CaMKII and PKCα *in vitro* as well as on the cell physiological level utilizing specific PKC inhibitors [26,31,32]. CaMKII, from either neuronal tissue or gastric mucosa, was demonstrated to be directly phosphorylated by PKCα [26,32]. In the case of gastric mucosal CaMKII, it was also shown that PKC-dependent phosphorylation of CaMKII reduced the phosphotransferase activity of CaMKII [32]. We observed that in gastric parietal cells each PKC-inhibitor, Gö 6976 or Ro 31-8220, positively affected the *in vivo* activity of CaMKII. These inhibitors became specific as, in the chosen cell system, PKCα was the only member of the cPKC subfamily found to be abundant [26]. Here we show that inhibition of PKCα activity diminished phosphorylation at the calmodulin-binding site, suggesting that PKCα partially contributes to phosphorylation at T305/306 of CaMKII and thus strengthening Ca^{2+}/calmodulin-independence of activated CaMKII [33]. PKCα-dependent attenuation of CaMKII phosphorylation at T286/287 was not observed to be additive for the suppressive activity of PP1 and PP2A. It was demonstrated elsewhere that both PP1 and PP2A dephosphorylate cisphosphorylation but

not transphosphorylation sites of PKC [34]. The PP2A regulatory subunit is transphosphorylated by PKCα, indicating a bidirectional, mutual transregulation of PP2A and PKCα [34]. These interactions help to explain why simultaneous blocking of PP1/PP2A and PKCα did not result in a synergistically induced hyperactivity of CaMKII present in TV.

SA contains predominantly autoactivated, autonomous CaMKII, which explains the already "adapted" medium level of activity [10]. Despite this fact, calcium is able to induce a short-term burst of phosphorylation in SA-CaMKII. The early phase before the peak of maximum phosphorylation at T286/287 of SA-CaMKII was much more robust towards any toxic perturbation besides KN-93, KN-62 or TPA, than TV-CaMKII. We assume that the robustness of SA-CaMKII phosphorylation is closely associated with the activity of PKCα, which may enhance the activity of PP1/PP2A. In essence, Ca^{2+}-dependent activation of SA-CaMKII activity for only effective if the activities of PKCα and PP1/PP2A were simultaneously blocked. This observation was confirmed for *in vitro* studies of SA-like plasma membranes of PKCα-deficient mice, suggesting the interdependent role of both PP1 and PP2A as well as PKCα during adaptation of CaMKII activity.

The kinetics of CaMKII phosphorylation in signalosomes can be divided into two phases (Fig. 3k), each of which seems to have its own function. The initial burst of CaMKII phosphorylation and, therefore, its activation has been observed to be stringently time-limited. It did not last more than 10 min with a maximal peak after 7 min of induction. A short-term peak of activity may be necessary to trigger Ca^{2+}-dependent downstream physiological events other than apoptosis. As recently reported, application of microcystin (0.5 to 2 μM) and CaMKII activity are both required for at least 11.5 to 16.5 min to induce detachment of cells and launch zeiosis as necessary steps leading to apoptosis [9]. Therefore, the observed initial peak of CaMKII phosphorylation, which lasted ≤10 min, did not appear to be sufficient to induce apoptosis. In the presence of PP1/PP2A inhibitor toxins, signalosome-associated CaMKII exhibited hyperphosphorylation for ≥10 min, which seems to be enough to trigger apoptosis [9]. After 15 to 20 min, microcystin-induced apoptosis became independent from CaMKII [9].

To assess the role of partitioning of CaMKII activity [11], we found a strong similarity between the kinetics of CaMKII phosphorylation *in vivo* compared to the kinetics of TV *in vitro*. Similarities were less obvious for the kinetics of CaMKII phosphorylation *in vivo* compared to SA *in vitro*. Thus, CaMKII of cytoplasmic TV appears to be more involved in the regulation of apoptosis than SA-CaMKII. CaMKII signalosomes of TV appear to represent a precisely tuned system designed to induce apoptosis in response to perturbed transregulation of CaMKII.

In summary, transregulation of CaMKII phosphorylations at T286/T287 and T305/306 of cytoplasmic TV signalosomes by PP1, PP2A, PP2B and PKCα stringently enables only short-term (≤10 min) burst activation of CaMKII regulatory sites, which is not sufficient to trigger apoptosis. Hyperphosphorylation for >10 min induced by PP1/PP2A-inhibitors such as CA or overexpression of constitutively active CaMKII leads increased apoptosis. Hence, transregulation of CaMKII admits a working CaMKII, which only supports apoptosis during perturbations of upstream signal transduction in its transregulators.

Acknowledgments

Work was supported by grants of Medizinische Hochschule Hannover (HiLFII) to MF. We are very grateful to Prof. Paul De Koninck (Université Laval, Québec, Canada) for mGFP-CaMKII vector constructs.

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